Ganoderma boninense basidiospores in oil palm plantations: evaluation of their possible role in stem rots of *Elaeis guineensis*

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Ganoderma boninense basidiospores in oil palm plantations: evaluation of their possible role in stem rots of *Elaeis guineensis*

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Basidiospores are implicated in the distribution and genetic diversity of *Ganoderma boninense*, cause of basal stem rot (BSR) and upper stem rot (USR) of oil palm (*Elaeis guineensis*). Measurement of aerial basidiospores within plantations in North Sumatra showed continuous and high production over 24 h (range c. 2-11,000 spores/m^3) with maximum release during early evening. Basidiospores applied to cut surfaces of fronds, peduncles and stems germinated *in situ*. Equivalent, extensive wounds are created during plantation harvesting and management and represent potential sites for formation of infective heterokaryons following mating of haploid basidiospore germlings. Notably, use of spore-sized micro-beads showed that basidiospores could be pulled up to 10 cm into severed xylem vessels, where they are relatively protected from dehydration, UV irradiation and competing microflora. Diversity of isolates from five locations on two plantations was assessed by RAMS fingerprinting. Isolates from within individual palms with USR were identical and represent single infections, but different USR infections had unique band patterns and reveal separate infections. Some BSR affected trees contained >1 isolate, thus had multiple infections. There was one example of adjacent BSR palms with the same isolate, indicative of vegetative spread, but there were no identical genets from BSR infections and adjacent fallen palms. Isolate diversity was as great within a plantation as between plantations. It is evident that basidiospores play a major role in spread and genetic variability of *G. boninense.* Evidence for direct basidiospore infection *via* cut fronds, indirectly through roots *via* colonized debris and less frequently, infection by vegetative, clonal spread is considered.

Keywords: white rot, host-pathogen interaction, sexual recombination, tetrapolar mating, DNA markers, pathogen dispersal

Introduction

Considerable yield losses and often death of palms continue to be inflicted on the oil palm (*Elaies guinensis*) in South East Asia and Papua New Guinea by the white-rot fungus *Ganoderma boninense* (Corley & Tinker, 2003; Pilotti *et al*., 2002; Rees *et al*., 2007). Basal stem rot (BSR) involves decay of the lower stem and sometimes the root system, leading to severe symptoms such as flattening of the crown and unopened spear leaves. Basidiocarps characteristically emerge from the lower stem. Upper Stem Rot (USR) is generally considered to be a less frequent manifestation although in some estates in North Sumatra, the incidence is increasing (unpublished data, LONSUM). USR as described here is defined as a decay of the upper stem and basidiocarp formation ≥2m above ground level. If affected palms are felled and dissected, USR infection is revealed to be unconnected to BSR; in severe infections, stem fracture can result (Hasan *et al*., 2005; Pilotti, 2005; Rees *et al*., 2007).

Elucidating the route of infection and extent of pathogen diversity is crucial in order to enable development of successful management practices for disease control. BSR infection can result from root infection, presumably following root contact with soil inoculum or other infected roots. Oil palm roots from mature palms can extend up to four planting rows, so root contact will be frequent (Miller *et al*., 1999). Rees *et al*. (2007; 2009) clearly showed that controlled root infection leads to typical symptoms and can occur in the field. In contrast, others have questioned the role of root infection, based on the high genetic diversity of *G. boninense* isolates (Miller *et al.* 1999; Pilotti *et al*., 2003).

Other root-infecting basidiomycetes such as *Heterobasidion annosum* and *Armillaria mellea* spread from tree to tree through soil by vegetative growth, often as a single genet (Woodward *et al*., 1998). However, genetic studies of *G. boninense* in Malaysia and Papua New Guinea reveal considerable diversity in oil palm plantations according to mitochondrial DNA markers, mating alleles and somatic (or vegetative) compatability (Miller *et al*., 1999; Pilotti, 2005). Infections by separate genotypes must have arisen through sexual recombination and subsequent dispersal *via* spread of

basidiospores. Pilotti *et al.* (2000) revealed the great diversity in monokaryons, based on RAPDs, such that no two isolates showed an identical RAPD genotype. Also, outbreaks of BSR in new plantations where *G. boninense* inoculum is not present in debris or soil, implies introduction by spores. Basidiospores have been detected in very high numbers under single basidiocarps (Sanderson, 2005). Outcrossing is favoured thanks to *G. boninense* being heterothallic and tetrapolar with multiple alleles at both mating type loci (Pilotti *et al*., 2002).

Monokaryotic mycelium from basidiospores can colonize palm wood but is non-infective (Rees *et al*., 2007); anastomosis with a compatible mating type is required to form the potentially invasive and faster growing heterokaryon. Basidiospore germlings readily anastomose (Pilotti, 2005) and mating could occur either on the palm surface, or during colonization of organic debris in soil (Flood, *et al.*, 2002). Nevertheless, many and various attempts to infect mature palms and seedlings with basidiospores have failed (see Hasan *et al*. 2005). Also we have previously demonstrated the very weak, competitive saprotrophic ability of *G. boninense* in soil and in the substantial organic debris that accumulates at the frond-stem junction of oil palms (Rees *et al*., 2007). Therefore the question arises as to where a heterokaryon might form and flourish to create a sufficient inoculum. It is important to note that infection of palm roots requires a substantial inoculum (Rees *et al*., 2007).

In an attempt to address some of these anomalies, here we quantify for the first time airborne basidiospore concentrations and their diurnal fluctuations in plantation air samples. We assess the capacity of basidiospores to germinate on and in potential wound sites on oil palms,

We extend the study of *G. boninense* genetic diversity by molecular characterization, using nuclear DNA markers, of isolates from BSR and USR infections and from isolates taken from fallen palms (those killed and left within established plantations) at five locations in North Sumatra. Few studies have addressed relationships between USR and BSR affected palms and fallen palms (FPs). FPs become heavily colonized with *Ganoderma* and may be an important source of inoculum. Also Sumatra potentially offers a different pathogen population from Malaysia and PNG.

Numerous molecular tools were considered, based on sensitivity, isolate discrimination, facilities and available skill levels and cost. Randomly amplified microsatellites (RAMS) was eventually chosen. RAMS has now been used in a

number of studies on fungal diversity including isolates of *Phlebiopsis gigantea* from Europe and North America (Vainio & Hantula, 2000; Vainio *et al*., 1998) and pine rusts (*Cronartium flaccidum* and *Peridermium pini*) (Hantula *et al*., 2002) This work shows the importance of basidiospores in dissemination and infection but suggests several mode(s) of infection are in operation.

Materials and Methods

Quantification of airborne spores within plantations

A Biotester® RCS centrifugal air sampler (Biotest UK) was used to sample the concentration of airborne spores within oil palm plantations at Bah Lias Research Station (BLRS), Sumatra. Water agar was loaded into the sampler which was run for 8 min per sampling, performed in the mid point of interrows and held at 2.0 m height. Agar blocks were then removed and observed microscopically for trapped spores. Four samples were taken for each time point and location to provide mean concentration according to: spores/m^3 air = no. basidiospores on agar strip x 1000/volume of air sampled (in an 8 min period 320 litres of air was sampled). It was not possible to differentiate spores of different *Ganoderma* species, which often have very subtle differences. Nevertheless, c. 90% of the *Ganoderma* basidiocarps within the plantations studied are from *G. boninense* (unpublished observations of the senior author); the characteristic morphology (basidiospore dimensions were 9.8 to 4.5 µM (mean), and reddish brown, narrow ellipsoid with a visible hilar appendage and often containing a large vacuole (see Fig. 3), characteristics of *G. boninense* (see Pilotti et al. (2004)) enabled easy discrimination from contaminating fungal spores of other genera and from some other basidiospores. *Ganoderma* basidiospores typically comprised c. 25% of the collected fungal spores.

Collection of basidiospores and preparation of spore suspensions

Basidiospores were collected from fresh basidiocarps of *G. boninense* emanating from trunks of mature, diseased oil palm. Spore collection was conducted between 06:00 and 10:00 h by placing Whatman filter paper, held in place by aluminium foil, directly

below the pores of active fruiting bodies. Spores were then air dried for 10-15 min. The spore-coated filter paper was then cut into small pieces and added to SDW (pH 5.5) to make a suspension. Spore concentration was calculated with a haemocytometer and in all cases spores were inoculated onto wounded oil palm tissues and also tested for viability *in vitro*, on the same day as collection.

Basidiospore viability was quantified microscopically after 48 and 72 h from 100 µl of 1 x 10^6 / ml spores spread on three replicate plates of water agar (pH 5.5).

Scanning Electron Microscopy

Samples (≤5mm maximum dimension) of palm tissues previously inoculated with basidiospores were collected and placed in 3.5% glutaraldehyde (Agar Scientific) in 0.05 M piperazine-*N*, *N* '-bis (2-ethanesulfonic acid) (PIPES) buffer at pH 8.0. Tissue was then cut into 3x3x1 mm pieces and, still immersed, exposed to vacuum for 16-20 h. Samples were then viewed with low temperature scanning electron microscopy (cryo-SEM) on a JEOL SEM6310 model scanning electron microscope fitted with an Oxford Instruments Cryotrans 1500 system attachment.

Spore germination on exposed tissue surfaces of oil palm

Germination of basidiospores, combined after collection from two basidiocarps on two spatially separated palms, was assessed on cut surfaces of oil palm fronds and peduncles. This exposed tissue represents the most extensive and frequent wound sites created in plantations as at harvest, fruit bunches are removed and fronds are also excised to allow access to the bunches. Also germination was determined within xylem vessels of fronds, severed 10–15 cm from the main stem, and on wounded trunk epidermis, which was cut to a depth of *ca.* 5cm.

Fronds and peduncles were cut near the stem junction by machete (ethanol washed) to leave a smooth, near-horizontal surface onto which 5 ml of a freshly prepared (<1 h) spore suspension (1x10^8 spores/ml) was applied immediately, or in some cases two weeks after wounding. The trunk outer layer was breached and a wedge excised to create a horizontal surface. Cut tissues were then covered with a plastic bag to prevent

removal of spores by rainfall and to maintain high RH. After 48 and 72h, treated tissues were excised with a scalpel and sectioned (transverse sections [TS] for surface examination and also longitudinal sections [LS] made to examine spores in xylem vessels) and fixed for subsequent analysis by cryo-SEM. For assessment of spores drawn into xylem, eosin dye (2 mg/ml) was added to spore suspensions. This method revealed the location of functional vessels and guided sectioning along the resulting red vascular tissue.

Five trees of the same age (5 years) and from the same plantation were used and one frond and peduncle from each palm was assessed for each day of the trial. Trunk wounds were made on five 15 year old palms.

Xylem vessel length

A suspension containing distilled water, eosin dye and spore-sized (2-20µm) fluorescent vinyl particles (Elgersma *et al*., 1972) was prepared and applied to the freshly cut surface of oil palm fronds. The suspension was added to the cut surface of three different length fronds at 15 min intervals maintaining an excess of suspension on the cut surface at least for 1 h. Thin hand-cut sections were prepared after 24 h every 1 cm using a razor blade and examined microscopically (Leica DMIRB microscope with fw4000 imaging software). Particles fluoresced bright red under incident light

Sampling of isolates.

Ganoderma isolates used for molecular characterisation were isolated from basidiocarps and necrotic tissue from BSR and USR affected palms from five plantings (plots) at two LONSUM owned estates located at 23 kms distance: Bah Lias and Sungei Bejanker. USR samples were taken from felled palms, which were selected for felling if they had evidence of USR 2m or greater from the base of the tree, with no evidence of BSR.

Isolates used for sequencing and fingerprinting (57 isolates) are listed in Table 1.

Plots were given a numerical code; the first two numbers are the year of planting and the three subsequent numbers represent the number of trees in the planting. Plots 86-200, 85-200 and 88-300 were from Bah Lias Estate and 84-300 and 86-400 were from Sungei Bejanker Estate. Mature plots were chosen so that incidence of BSR would not be limiting with numerous fallen palms located adjacent to standing BSR infections. Essentially sampling involved obtaining isolates from adjacent palms which had a fallen palm as the focus. Thus in Table 1, isolates coded T1, T2 or T3 would be from three adjacent trees.

One BSR palm was felled in each plot and isolation was attempted from rotting tissue and basidiocarps using a *Ganoderma*-selective medium (GSM) as described by Rees *et al*. (2007). Other than antibacterial components, antimicrobial components comprised (g/l) pentachloronitrobenzene (285), Ridomil (130), Benlate (150) and tannic acid (1.25). Sampling was also attempted from host tissue and basidiocarp ground tissue from at least one adjacent fallen palm in each plot and from basidiocarps on any nearby BSR infected palms. These palms were not felled because of financial considerations and therefore sampling from rotting tissue was not possible. One USR tree was also felled from each plot with sampling from tissue and basidiocarps.

Success of *Ganoderma* isolation from palm tissue was not high and was particularly low from fallen palms, but was most easily obtained from basidiocarps. Initial isolation was facilitated by use of GSM. Isolates were then subcultured onto PDA before extraction for DNA, sequencing and fingerprinting as described above. Multiple isolates of the cultures were stored on PDA on slants covered with sterile mineral oil or sterile water at room temperature and refrigerated at ≤6ºC.

Harvesting *G. boninense* mycelia and DNA extraction

Ganoderma isolates were grown on PDA for 1 wk. 1 cm^2 plugs were taken from the leading edge of the mycelium, placed in 60 ml of 3% malt extract (Oxoid) in 250 ml conical flasks then incubated at 28ºC on a rotary incubator at 120 rpm, for 4-5 d. Mycelium was then removed, filtered and washed in SDW, then frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle. 100 mg of the powder was then used for DNA extraction and the remainder was stored at -70ºC for future extractions.

DNA extraction was achieved from 57 isolates using the DNeasy® plant DNA extraction kit (Qiagen) as described in manufacturer's instructions.

PCR

Universal fungal rDNA primers ITS1 (5' TCCGTAGGTGAACCTGCGG) and ITS4 (5'TCCTCCGCTTATTGATATGC) were used to amplify the ITS1, 5.8S rDNA gene and ITS2 region of *G. boninense* yielding a product of approximately 650 bp (Latiffah *et al*., 2002). PCR amplification conditions are described under Supplementary Information.

Sequencing

Sequencing reactions were performed in 5 µl volumes using 96 well PCR plates (AB Gene) according to manufacturer's instructions for Bigdye® (Applied Biosystems). Twelve millilitres of milliQ water was added to each well, sealed, vortexed and centrifuged at 400 x g for 20 sec. Fifty-two microlitres of absolute ethanol and 3 mM sodium acetate (50:2 v/v) was then added to each well and mixed. The plate was then chilled in a -20ºC freezer for 30 min before centrifugation at 1350 x g for 30 min. The plate was then blotted onto paper tissue and centrifuged inverted on paper tissue for 20 sec at 200 x g. 150 µl of 70% ethanol was added to each well, sealed and centrifuged at 1350 x g for 30 min. After centrifugation the plate was blotted on papaer tissue and centrifuged inverted on paper tissue at 200 x g for 20 sec. The plate was then air dried and sealed before sequencing.

DNA Fingerprinting

Fingerprinting of *G. boninense* was carried out using randomly amplified microsatellites (RAMS) as described (Dai *et al*., 2003). Degenerate primers 5'DHB$(CGA)_5$ and 5'HBH$(GAG)_5$, where D=A/G/T, H=A/C/T, B=C/G/T, were used to amplify microsatellite DNA. PCR amplifications were as above with an initial denaturation of 10 min at 95ºC followed by 37 x [30 sec denaturation at 95ºC, 45 s

287 annealing at 61ºC] and 2 min extension at 72ºC, followed by a final 10 min extension
288 at 72ºC. 20 µl of the reaction mixture was analysed on a 2% w/v agarose gel.

289

290 **Statistical Analysis**

291

292 At least three RAMS amplifications were performed on separate occasions using the
293 same DNA sample for each *Ganoderma* isolate and only amplicons that reproduced
294 consistently were scored for presence (1) or absence (0). Identical banding patterns
295 were regarded as genetically identical and were only introduced to the matrix once for
296 statistical analysis. The analysis is shown in Supplementary Information.

297

298

299 # Results

300

301 **Quantification of airborne spores within plantations**

302

303 Accurate quantification of basidiospores in plantation air combined with circadian
304 influence on spore release by *G. boninense* has never previously been determined.
305 Four successive 8 min air samples (which were considered replicates) were taken with
306 a Biotester® positioned at 2m height in the early morning, midday, early evening and
307 midnight over a 4 day period and mean basidiospore no./m^3 was determined as
308 described above.

309 Basidiospores were detected in high numbers throughout 24 h periods, but greatest
310 spore release occurred in the early evening (Figure 1). Basidospore density was lowest
311 at 07.00 h with mean ca. 2,000/m^3, doubling by 12.00 h, then peaking at 19.00 h with
312 ca. 11,000 spores/ m^3 and declining by 24.00 h to c. 4,500 spores/ m^3.

313 Samples collected with the Biotester® at a distance of 10 cm beneath young, active
314 brackets with a pore surface of approximately 10 cm^2 from 07:00 h to 23:00 h
315 revealed a basidiospore release rate of *c.* 140,000 spores/min.

316 Airborne spore concentrations are likely to depend upon abundance of basidiocarps,
317 which will reflect presence of infected and dead palms.. Therefore air samples were
318 also taken at 12.00 h (on successive days under rain-free conditions) from 8 and 17

year-old plantings and from a replanted area containing windrowed trunks felled 3 years previously.. Windrowing involves uprooting of previous bole and trunk tissues and stacking these along the inter-rows (Virdiana *et al*., 2010). Mean basidiospore concentrations were greatest in the oldest planting (c. 4,500 spores/m^3 (SE 507)), lower in the 8 year stand (c. 3,000 spores/m^3 (SE 1014)) and lowest in the windrow samples (c. 2,000 spores/m^3 (SE 725)), but differences were not significant (one-way ANOVA ($P < 0.239$, df = 2).

***In vivo* spore germination**

In order to mimic possible infection conditions, i.e. encourage anastomosis and formation of potentially infective dikaryons, basidiospores from two different basidiocarps on spatially separated, infected trees within a mature oil palm block, were mixed and applied concurrently to the cut host surfaces. Basidiospores were added immediately to cut surfaces or to surfaces two weeks after wounding (3 replicates per treatment).

Spores germinated readily on water agar (pH 5.5) with germination ranging from 57-85% (data not shown). Germination *in vitro* was not significantly different after 72 h than 48 h, or when suspended in eosin dye (used later to reveal functioning xylem vessels). Germination sometimes varied with spores from brackets on spatially separated palms and in one experiment appeared greater when the spore suspensions were mixed (data not shown), but differences were not significant (Tukey-Kramer HSD p = 0.05). Thus spores were viable at the time of inoculation of palm tissues.

Basidiospores germinated readily on wounded surfaces in the field (Figure 2). All wounded surfaces: peduncles, trunk tissue and fronds, supported very high germination rates. Some basidiospores had been pulled into xylem vessels and germination was similarly high to that on the exposed frond surface. Wounded trunk tissue and peduncles showed markedly more microbial contamination than frond tissue and were more difficult to analyze microscopically. All the surfaces left for two weeks after wounding became colonized with diverse microorganisms and were more difficult to assess basidiospore germination.

351

352 **Vessel lengths in oil palm fronds**

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354 Severed xylem vessels are exposed at pruning cuts to fronds and petioles and
355 represent a considerable surface area for possible infection. As a result of negative
356 tension in xylem, solutions and suspensions will be withdrawn into vessels from the
357 cut surface. This offers a potential route for basidiospores to penetrate deep within the
358 palm. The extent of this access will be dictated by the length of vessels, which
359 terminate at end walls and function to limit the progression of embolisms and particles
360 such as fungal spores (Cooper, 1981).
361 Eosin dye applied to a wounded frond surface allowed visualisation of functional
362 xylem vessels and progressed >20 cm into the tissue, but as a solution it is not
363 restricted by the presence of end walls (Figure 3a). In order to determine xylem vessel
364 length, and therefore how far a basidiospore may be pulled into wounded fronds, red
365 vinyl, spore-sized fluorescent particles were mixed with eosin and applied to cut
366 surfaces. UV microscopy of sections revealed red particles were withdrawn up to 10
367 cm (Figure 3b). Frequency of particles was greatest nearest the cut surface, showing
368 that frond vessel lengths comprise a variable population but do not extend beyond 10
369 cm. Figure 3c shows vessels terminating (end walls) in a longitudinal section made
370 near a cut frond surface.
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372 .
373 **Sequencing of *Ganoderma* ITS1, 5.8S rDNA and ITS2**

374

375 Sequencing confirmed the identity of most of the isolates as *G. boninense* after
376 BLAST analysis using the NCBI database. Previous sampling of *G. boninense* in
377 Indonesia by Utomo *et al.* (2005) and numerous species of the *G. lucidum* complex by
378 Moncalvo *et al* (1995 a; b) provided many sequences for comparison.
379 All isolates obtained from BSR and USR infected standing palms were found to be
380 *G. boninense*; however sequences from several isolates from FPs revealed different
381 *Ganoderma* species. For example, isolate FPA B1 S2 from plot 85-200 had most
382 homology to *G. fornicatum* isolates from Taiwan and FPC R4 S2 was most closely
383 related to *G. gibbosum* from mainland China. Thus, in this study, only *G. boninense*

was found to cause infection of oil palm whilst other *Ganoderma* species were saprotrophs of fallen *palm tissue*. It should be noted that other spp. of *Ganoderma* especially *G. zonatum* and *G. tormatum*, have been linked with palm diseases, but they were not revealed in this analysis. Complete identity of the 5.8S rDNA was observed in all isolates from Sumatra and also with *G. boninense* isolates from oil palm in Indonesia (Utomo *et al*., 2005). ITS1 and ITS2 are more variable than 5.8S rDNA and have been used for numerous interspecific phylogeny studies (Moncalvo et al., 1995b, Smith & Sivasithamparam, 2000). Three residues in ITS1 and one residue in ITS2 showed variability, but this was not sufficient for determination between individuals (data not shown). Inability to differentiate between closely related isolates was also observed by Latiffah *et al.* (2002) using RFLP of ITS1 and ITS2 on populations within oil palm and coconut. For greater discrimination, randomly amplified microsatellites (RAMS) was used to fingerprint isolates.

Randomly amplified microsatellites (RAMS)

Fingerprinting based on patterns of similarity in RAMS profiles of isolates was conducted to address key questionss relating to infection, spread and diversity of *G. boninense*: i) Are neighbouring palms infected by the same isolate of *G. boninense*? ii) Is the same isolate of *G. boninense* found in palms and adjacent fallen palms? iii) Are BSR and USR infections in a palm the result of a single infection event and thus contain only one *G. boninense* genet? iv) What is the extent of diversity within plantations and are distinct populations found on different plantations?

The RAMS amplification adapted from Hantula *et al*. (1996; 2002) provided 6-12 clear bands per amplification, ranging from 400-1500 base pairs for each *G. boninense* isolate. Gel images were scored manually and identical band patterns were regarded as the same genet; a binary matrix was compiled from the different profiles (Table 2). This technique was reproducible and revealed differences between isolates that were inseparable by ITS sequencing.

Examination of the RAMS profiles did not reveal any identical genets between BSR infected trees and FPs whether adjacent or from any of the plots (Figure 4a). Therefore, there was no evidence to indicate secondary vegetative spread of the disease from FPs to neighbouring palms.

Only one plot contained two adjacent palms infected with BSR that appeared to have identical fingerprints. BSR T4 B1 had an identical band pattern to BSR palm BSR T3 B2 in plot 86:400 from Sungei Bejanker Estate (Figure 4b); in all other cases RAMS profiles from isolates obtained from separate palms were unique, indicating infection by separate genets.

Isolates obtained from individual USR infections i.e. from different palms, each had unique band patterns, but within each palm infected by USR, the RAMS banding profile was identical (Figure 4c); this pattern suggests that a single infection event causes USR infections.

In contrast, in three of the seven BSR infected palms more than one RAMS profile was evident: Isolates BSR T1R1 and BSR T1 R2 had distinct banding patterns within an infected palm (plot 88-300), BSR T3 B2 was distinct from other isolates within the same palm in (plot 86-400) and BSR T2 B2 was unique from other isolates within the same palm (plot 86-200). This indicates that multiple infections involving more than one isolate can occur within a single palm affected by BSR. However, Fig. 4d reveals that seven isolates from a single BSR infected palm show the same RAMS profile, indicating that in this instance isolates were clonal.

Examination of the matrix using cluster analysis showed that individuals within a single planting did not cluster together more than those from different plantings (Figure 5). In addition, isolates from Sungei Bejanker were equally likely to cluster with isolates from Bah Lias estate as they were to cluster with those within the same estate. For example, *G. boninense* isolates obtained from palm BSR T2 in plot 86-200 in Bah Lias, have more similarity (based on number of shared bands) to isolates from fallen palms FP T1 & T2 in Sungei Bejanker plot 84-300 than to other isolates from Bah Lias. . The cluster analysis also confirmed that the only identical isolates from neighbouring palms are BSR T4B1 and BSR T3B2 in plot 86-400.

Discussion

Evidence based on the physical and genetic discontinuity of USR from BSR infections and especially the high genetic diversity of *G. boninense* isolates within plantations, even between most neighbouring trees with BSR, suggests that basidiospores play a key role in the development of both manifestations of stem rot.

This is the first record of temporal quantification of basidiospore release in air samples from a plantation. Basidiospores are produced in prolific numbers throughout the sampling period, with maximal release in the evening, earlier than the midnight maximum release reported by Ho and Nawawi (1986). Clearly there will be constant potential inoculum to colonize wounds and palm debris throughout the plantation. Previous assessments of basidiospore release were restricted to individual basidiocarps, when production was estimated by Sanderson (2005) at *ca*. 2 million spores per minute from a 5 cm^2 bracket. Similarly, we detected mean *ca*. 1.4 $x10^5$ spores per minute from 10 cm^2 pore surface area during daylight hours.

In spite of the evidence for their apparent involvement, there have been no successful attempts at infecting oil palm with basidiospores (Hasan *et al*., 2005; Idris pers. comm; Thompson, 1931; Yeong, 1992 cited in Miller *et al*., 2000). This presumably reflects the relatively low aggressiveness of *G. boninense* and the need for large inoculum, as discussed by Rees *et al.* (2007). Some other fungal tree pathogens can infect, directly or indirectly by spores, such as *H. annosum via* conifer stumps (Woodward *et al*., 1998) and *Cryphonectria parasitica via* wounds in chestnut bark (Nuss, 1992).

The potential for infection sites in plantation palms is considerable, with extensive wounds created by routine harvesting (severing the fruit bunch peduncle) and pruning (of frond base to free the fruit bunch). Also trunk wounds are more likely in older palms as harvesting becomes more difficult at greater height. Here we show for the first time that basidiospores can germinate abundantly on cut surfaces under plantation conditions. Spores contaminating cut frond surfaces are withdrawn into xylem as a result of negative tension within functional vessels (Cooper, 1981). The potential distance of ingress is ca. 10 cm, reflecting vessel length, as dictated by vessel end walls (Cooper 1981). Here, basidiospores would be relatively protected from dehydration, microbial competition and solar radiation. Spores readily mate according to Pilotti (2005) and anastomosis was apparent *in situ* from our cryo-SEM images. Resulting heterokaryon formation, a prerequisite for formation of infective mycelium, could result in a lesion which extends into the palm trunk.

Some workers have implicated or provided indirect evidence for trunk infection *via* wounded surfaces of fronds. Initially, Thompson (1931) surmised that spores entered stem through old leaf bases or through pruning wounds. Sanderson & Pilotti (1997) cut back the rachis of decayed frond bases and followed lesions into the stem base.

Following stem expansion this initial infection would appear to have originated near the centre of palm base. Panchal & Bridge (2005) using PCR primer GanET detected *Ganoderma* in frond base material (sampling at 0.25-1 cm depth) with 73% of detections from the most recently pruned fronds, as might be expected in view of the considerable number of aerial basidiospores. 71% of detections were from frond bases near ground level where palms were beginning to show BSR symptoms, with the remainder on upper frond bases. Infection of wounded frond surfaces can occur according to Lim *et al*. (1992), but *G. boninense*-colonised oats was used as the inoculum source. In contrast, Hasan *et al*. (2005) failed to reproduce USR, even using *Ganoderma*-infested rubber wood.

USR is not linked to BSR (Hasan *et al*., 2005; Pilotti, 2005). RAMS analysis of *G. boninense,* showed individual USR infections only contained a single isolate of *G. boninense* and each isolate was genetically distinct. This pattern would be expected if infections in upper stems are exceptional events and derive from basidiospores. Pilotti (2005) recorded USR occurrence in only 0.01% of trees in PNG

Some BSR infections derive from root infection, as clearly evidenced by Rees *et al*. (2007). The inoculum might derive from contact with infected roots from neighbouring palms or from colonized debris. Infection of seedlings can occur from nearby colonized oil palm trunks with those seedlings nearer to the colonized trunks became diseased more quickly (Flood *et al*., 2005). Seedlings also become infected when planted near infected stumps from the previous planting (Hasan & Turner, 1998). Infection at replanting from colonized debris or from windrows remaining in the field is suggested by the reduced infection following fallowing and various windrow treatments (Virdiana *et al*., 2010), or when increased incidence of infection occurs following poor land preparation with infected boles left in the ground (H. Foster, pers. comm.).

A pattern of expanding clusters of affected palms might be predicted from root to root spread. There is only one such published report (Singh, 1991), although field observations in Malaysia still suggest its occurrence, with pattern dependent on first generation or replanting and if clean clearing has been practised (G S Thind, pers. com.). *Ganoderma* stem infections of amenity palms including oil palm, in Singapore, showed no obvious clustering and it was concluded that basidiospores are the means for dissemination and infection (Lim & Fong, 2005). Also recent GPS positioning of BSR-infected palms shows mostly random distribution of BSR in several estates in

519 Sabah (N. Hisham, pers. com.). Based on genetic diversity of isolates, some consider
520 that root infection and secondary vegetative infection between trees is of minor
521 importance (Miller *et al.* 1999; Ariffin *et al*., 1996; Pilotti *et al*., 2003).
522 Our data partly concur with isolate diversity in BSR, as three of seven affected
523 palms contained more than one isolate of *G. boninense*, based on RAMS profiles.
524 Likewise, a Malaysian study by Miller *et al.* (1999) showed six of eight BSR palm
525 isolates had different somatic compatibilities and mtDNA RFLP profiles, and Pilotti
526 (2005) found multiple isolates in single palms. This pattern clearly indicates multiple
527 infections rather than clonal spread. Nevertheless, clonal colonization can occur, as
528 revealed by all seven isolates from a single BSR infected palm with an identical
529 RAMS profile.
530 Molecular evidence for mycelial spread of the disease from FPs or BSR infected
531 palms to neighbouring palms was not strong in this study. RAMS profiles did not link
532 BSR infected trees to neighbouring FPs. Similarly, none of the band patterns from
533 FPs was shared with isolates from adjacent FPs. The data reveal a genetically variable
534 population in North Sumatra and the importance of basidiospores, which concurs with
535 previous studies in Malaysia (Miller *et al.*, 2000) and PNG (Pilotti, 2005; Pilotti *et al.*,
536 2003). However, isolates from within two adjacent BSR infected palms (plot 86:400,
537 Sungei Bejanker) did share identical profiles concurring with findings of Miller *et al.*
538 (2000) where adjacent BSR palm isolates displayed the same mtDNA RFLP band
539 pattern. Pilotti (2005) also obtained 2/15 isolates from adjacent palms with the same
540 somatic compatability. Therefore vegetative spread of the pathogen does occur.
541 However, these apparently conflicting mechanisms are not mutually exclusive.
542 Rees *et al*. (2007) showed multiple, natural infections of different roots in a single
543 palm and this could explain some of the diversity of isolates within BSR lesions. It is
544 tenable that, based on the continual spore deposition in plantations and the extent of
545 potential substrates, that a single trunk may become colonised with genetically
546 distinct isolates. Colonisation of woody substrates by diverse genotypes resulting
547 from basidiospores was demonstrated for pine root rot (*H. annosum*) and the
548 biocontrol fungus *Phlebiopsis gigantea*. Colonization of a single pine stump by
549 several genetically distinct individuals of *P. gigantea*, was based on morphological
550 characters, pairing experiments and RAMS fingerprinting (Vainio *et al*., 2001).
551 Similarly, Swedjemark & Stenlid (2001) isolated 27 genets of *H. annosum* from
552 within a single pine stump over two years based on somatic incompatibility studies.

553 The extent of variation within and between plantations is considerable and must
554 reflect the tetrapolar mating system of *G. boninense*, which provides inbreeding
555 restriction of 25% thus ensuring the pathogen acquires maximum diversification.
556 Once dikaryons are formed, they maintain their integrity such that isolates found in
557 plantations are individuals and will remain so. Cytoplasmic and nuclear exchange is
558 prevented by somatic incompatibility mechanisms (Pilotti *et al*., 2002). Cluster
559 analysis conducted on the binary matrix produced from banding patterns showed that
560 isolates from a single plot often did not cluster together more than those from
561 different plantings or even from a different estates at 23km distance. These data agree
562 with the high genetic variability observed in Malaysia and PNG. For example Miller
563 *et al*. (1999) identified 34 of 39 from one plot as distinct somatic incompatibility
564 groups. Sexual compatibility studies revealed great variation within an area studied in
565 PNG by Pilotti *et al.* (2003), with 81A and 83B mating alleles identified, and more
566 genetic relatedness between isolates 15-17 km distant than between adjacent
567 individuals. Somatic incompatibility studies yielded the same conclusions. Pilotti
568 (2005) suggests that migration of spores from outside planting areas explains how
569 new alleles are being detected every year.
570 Commercial oil palm is propagated as tenera seed produced from crosses between
571 dura x pisifera; thus, other than the small proportion of clonal palms planted, these
572 segregating populations present a heterogeneous host. Such heterogeneity could create
573 additional selection pressure for *G. boninense*, which is ideally designed through out-
574 crossing and prolific propagule generation to segregate and adapt for aggressiveness
575 traits (Miller *et al*., 2000; Sanderson & Pilotti, 1997).
576 In summary, a model is emerging of multiple modes of infection by *G. boninense*.
577 However, infection based on initial substrate colonization conflicts with the very
578 weak competitive saprotrophic ability of *G. boninense* in soil and organic debris,
579 shown by Rees *et al.* (2007). Not only does the ability of spores to infect wounds need
580 to be fully investigated, but so too does their capacity to colonize palm wood as felled
581 trunks or as remaining debris in soil.
582 Whilst disease control by the development of resistant material and methods to
583 reduce inoculum at replanting must continue to be pursued, where practicable,
584 management strategies should ideally include routine removal of basidiocarps (Hunt
585 & Pilotti, 2004).

Whilst disease control by the development of resistant material and methods to reduce inoculum at replanting must continue to be pursued, management strategies involving routine removal of basidiocarps could be investigated in some circumstances, as recommended by Hunt & Pilotti(2004). In first plantings with low levels of infection and few basidiocarps this should be beneficial, but this option might become impracticable in some second and third plantings with high basidiocarp frequency, as often found in Sumatra.

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720
721 **FIGURE LEGENDS**
722
723
724 **Figure 1. Diurnal fluctuation of aerial basidiospore numbers in a 17-year old**
725 **plot.** Error bars represent standard deviation of the mean of four samples at each time
726 point over 4 d, constituting 16 readings.
727
728
729 **Figure 2 a-f. Germination of *G. boninense* basidiospores on wounded fronds,**
730 **peduncles and trunk tissue.** a, b Germination of *Ganoderma* basidiospores on and in
731 cut frond (petioles) parenchyma cells. c. Basidiospore germination in xylem of cut
732 frond. d. Spore germination on cut fruit bunch stalks (peduncles). e. Mass of
733 germinating spores with germ tubes and hyphae in very close association (apparent
734 anastomosis), on wounded trunk surface. f. Initial stages of basidiospore germination
735 on wounded trunk surface. Note the characteristic basidiospore morphology with the
736 truncated apex. All are cryo-SEM images and show spores 48 h post inoculation to
737 wounded surface. Scale bars represent 10 µM. Germination of these spores *in vitro*
738 was ≥57%.
739
740 **Figure 3 a-c. Vascular anatomy and length in oil palm fronds revealed by**
741 **fluorescent particles and eosin dye.** a. Longitudinal section of cut frond showing
742 functional xylem vessels stained with eosin applied immediately to the cut surface. b.
743 Red fluorescent particles (arrow) within a xylem vessel ca. 10 cm below a cut surface
744 reveal the maximum vessel length, based on passage distance of particles unable to
745 traverse vessel end walls. c. LS of frond showing termination of a wide xylem
746 vessel, and a narrower adjacent vessel. Pit fields are evident as the vessels tapers to
747 end wall (arrows). Spores (and fluorescent particles, see Fig 4 b) would be trapped
748 here. d. Vascular bundle in control frond, transverse section showing arrangement of
749 xylem vessels. Scale bars represent 100µM.
750
751 **Figure 4 a-d**. **RAMS profiles of *Ganoderma boninense* isolates.**

Band sizes were estimated with a 100bp ladder (Invitrogen); the 3 brightest markets show 600, 1500 and 2000 bp. For a-c, two lanes represent two PCR reactions for a single *G. boninense* isolate.

Gel images are representative of a cross section of the 57 isolates; images from isolates not included here were satisfactory for reading and translating to the binary matrix.

(a) Microsatellite profiles from a single BSR infected palm and two adjacent fallen palms from plot 85-200. Lanes 1&2 = BSR T2B1, 3&4 = BSR T2B2, 5&6 = FPA T1R1, 7&8 = FPA T1R2.

(b) Microsatellite fingerprints from adjacent BSR infected palms in plot 86-400 and four FPs from plot 84-300. Lanes 1&2 = BSR T4B1, 3&4 = BSR T3B3, 5&6 = FP T1B1, 7&8 = FP T1B3, 9&10 = FP T2R2, 11&12 = FP T2B1.

(c) Fingerprints of *G. boninense isolates* from a USR infected palm and fallen palms in plot 84-300. Each lane is the result of a PCR amplification. Lane 1 = FP T2R1, 2 = FP T2R2, 3 = FP T2R3, 4 = FP T2B1, 5 = USR T1R1, 6 = USR T1R2, 7 = USR T1R3, 8 = USR T1R4, 9 = USR T1R5

(d) Seven *G. boninense* isolates from a single BSR infected palm from plot 85-200 are identical. Lanes 1&2 = BSR T1R1, 3&4 = BSR T1R2, 5&6 = BSR T1R3, 7&8 = BSR T1B1, 9&10 = BSR T1B2, 11&12 = BSR T1B3.

Isolates were from: FP-fallen palm; BSR basal stem rot; USR upper stem rot; R rot; B basidiocarp

Figure 5. **Hierarchical clustering of *Ganoderma* isolates.** Clustering produced using *pvclust* in R. Distances were binary (based only on shared presences, not absences), and clusters were formed using average linkage. Numbers above internal nodes indicate approximate *p*-values (%) based on 10,000 bootstrap re-samplings of characters. The first (left) number is the Approximately Unbiased (AU) value, and the second (right) is the conventional Bootstrap Probability (BP) value. AU values are computed using multiscale bootstrap re-sampling, and constitute a better approximation to unbiased *p*-values than those obtained using conventional bootstrapping (Schimodaira, 2004). Values are consistent with expectations for the modest ratio of variables to objects (isolates) (6:5). Further details and references are in Supplementary information